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Monitoring adrenal activity in dairy cows under various feeding regimens using faecal glucocorticoid metabolites

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ABSTRACT

Adrenal activity in cattle can be measured by changes in plasma glucocorticoid concentrations. Cows treated with synthetic adrenocorticotrophic hormone (ACTH) to stimulate the adrenal cortex exhibited elevated plasma cortisol concentrations for 4-5h. Concentrations of faecal glucocorticoid metabolites (FGM) peaked between 8-9.5h after ACTH treatment, 2.5 fold higher than basal ($P<0.05$) and remained elevated for 13h (range, 11.1-16.1h). Faecal glucocorticoid concentrations were monitored in two strains of Holstein Friesian (HF) dairy cows farmed in New Zealand under two systems: NZ pastoral and North American concentrate-based. The overseas (OS) and New Zealand (NZ) HF were fed a pasture-based diet (Grass) or a total mixed ration (TMR) throughout lactation. Faecal samples were collected on four occasions during lactation. Faecal glucocorticoid concentrations of the cows fed Grass were consistently higher ($P<0.05$) than cows fed TMR. There was also a significant genotype effect; FGM concentrations of the NZ HF cows were higher than OS HF cows ($P<0.05$). In a separate study, no obvious trends were evident in FGM in NZ HF grazing pasture at three stocking rates (2.2, 3.2, 4.3 cows per hectare). Measuring FGM can be used to monitor acute adrenal activity in cows, but is more difficult to interpret as a measure of chronic stress.

Keywords: dairy cows; faecal; cortisol; ACTH; stress.

INTRODUCTION

Glucocorticoids are secreted by the adrenal gland in response to the hypothalamic-pituitary-adrenal (HPA) axis being activated by a stressor. Primarily an adaptive mechanism, glucocorticoids assist the animal in coping with stress by mobilising body reserves and regulating inflammatory responses to injury. However, sustained or long-term increases in HPA activity are thought to be detrimental (e.g., decreased growth and milk production, suppressed immune system function). Plasma cortisol has historically been used to assess animal stress (Broom & Johnson, 1993), however, excreted faecal and urinary cortisol metabolites can also be used to monitor adrenal activity in cattle (Möstl *et al.*, 1999; Palme *et al.*, 1998, 2000; Morrow *et al.*, 2000). Metabolites are excreted after metabolism and conjugation in the liver, and enter the intestine with bile and are integrated with digesta, undergoing further metabolism by gut microflora. The effects of acute adrenal activation in cattle (e.g., adrenocorticotrophic (ACTH) hormone challenge, transport) can be detected by increased faecal glucocorticoid metabolites (Palme *et al.*, 1998, 2000). Faecal sampling reduces the physiological/psychological stress associated with other procedures (i.e., restraint, venipuncture). Monitoring of faecal stress metabolite concentrations in livestock during routine management procedures may provide a useful method for quantifying standards of animal welfare. The objectives of this study were to (1) confirm the usefulness of the technique for monitoring adrenal activity in the lactating dairy cow following an ACTH challenge and (2) characterise faecal glucocorticoid metabolite concentrations in dairy cattle farmed in NZ under a wide range of systems (varying in genetic background, feeding regime, stage of lactation and stocking density).

MATERIALS AND METHODS

All experiments were conducted on dairy cows at Dexcel Ltd. (formerly Dairying Research Corporation Ltd.) during

the 1999/2000 lactation, with the approval of the Ruakura Animal Ethics Committee.

Study 1: ACTH Challenge

Details of the experimental design and blood sampling have been reported previously (Morrow *et al.*, 2000). Briefly, five lactating Holstein cows (542 ± 14.9 kg live weight; 59.4 ± 3.5 d post partum, range 54-73d) were housed in a semi-enclosed barn facility for a 5-day period in the spring (October). On Day 3, each cow received two injections of ACTH₍₁₋₂₄₎ (0.05mg i.v.; Synacthen, Novartis Pharma AG, Basle, Switzerland) administered via an indwelling jugular catheter at 0900 and 1100h, using a protocol that reliably elevates plasma cortisol concentrations for 4-6h (Verkerk *et al.*, 1994; Stelwagen *et al.*, 1998). Morning faecal samples were collected daily on Days 1-3 (pre-ACTH treatment) and a subsample from all spontaneous defecations was collected for the period from 8 to 45 h after the initial ACTH injection.

Study 2: Comparison of Holstein Friesians in pastoral or concentrate system

Genotype, diet and genotype x diet interactions were investigated in an ongoing experiment that is comparing HF genetics of New Zealand (NZ) or overseas (OS; North American and Dutch) origin grazing pasture/pasture supplement (Grass) or fed a total mixed ration (TMR). For additional details of experimental design refer to Kolver *et al.* (2000). The four treatments in this 2x2 factorial experiment were NZ Grass (n=14), OS Grass (n=13), NZ TMR (n=14) and OS TMR (n=14). Faecal samples were collected on three consecutive days on four occasions during lactation in September (early lactation), October (peak lactation), January (mid lactation) and March (late lactation) equivalent to 33, 73, 163 and 237 days in milk, respectively.

Study 3: Effect of stocking rate in a pastoral system

Faecal samples were collected from 56 lactating NZ

dairy cows grazing pasture on one of three farmlets, varying in stocking rate, 2.2 (LOW), 3.2 (MID) and 4.3 (HIGH) cows per hectare. Cows (15-19 per farmlet) were sampled on three consecutive days on four occasions: June (non-lactating/dry), September (early lactation), January (mid lactation) and April (late lactation).

Immunoassays

Faecal samples were collected fresh, stored on ice until freezing (-20°C), before being freeze-dried and crushed. Samples collected over three consecutive days were analysed individually. To extract, ~60mg of well-mixed powdered faeces was suspended in 80% methanol (5ml) and vortexed for 30mins. Following centrifugation, the supernatant was diluted 1:10 in buffer and stored frozen.

Faecal glucocorticoid metabolite concentrations were measured using the commercially available I^{125} radioimmunoassay kit (Rats & Mice Corticosterone kit, ICN Pharmaceuticals, NY, USA). Extracted faecal samples were diluted 1:10 in PBS (phosphate-buffered saline, pH 7.4) and analysed in duplicate. Standards (100 μ l) were pipetted to yield 3.125, 6.25, 12.5, 25, 50, 100, 250, 500, 1000ng/ml. Samples (100 μ l) were pipetted and 100 μ l steroid diluent added to the sample tubes. Standards, samples and controls were incubated with I^{125} labelled corticosterone (100 μ l) and anti-corticosterone antibody (100 μ l) at room temperature for 2h. Precipitant solution (250 μ l; PEG and goat anti-rabbit gamma globulins in Tris buffer) was added to each tube and vortexed. Tubes were centrifuged (15mins, 1000g), the supernatant decanted and the precipitate counted in a gamma counter. The antibody (raised in rabbit against corticosterone-3-carboxymethyloxime:BSA) cross-reacted with corticosterone 100%, desoxycorticosterone 0.34%, testosterone 0.1% and cortisol 0.05%. The inter- and intra-assay coefficients of variation for faecal control samples were 4.3 and 6.7%, respectively. Results are expressed as nanograms metabolite per gram dry faeces.

Plasma cortisol concentrations were measured in duplicate using a commercially available I^{125} radioimmunoassay kit (Coat-a-Count Cortisol, Diagnostic Products Corporation, Los Angeles, CA, USA). Sensitivity of the assay was 0.5ng/ml and the inter- and intra-assay coefficients of variation for bovine plasma were 8.0 and 6.6%, respectively.

Data Analyses

Results of the ACTH challenge are reported as mean \pm SEM, mean basal concentrations were calculated from the -30min and time 0 samples on Day 3. Concentrations were compared using Student's t-test for paired means. For the samples collected over each 3-d period in the farm systems studies (Studies 2 and 3) concentrations for each cow were averaged over the three samples before statistical analysis. Data from Studies 2 and 3 were analysed using the general analysis of variance procedure of Genstat (Release 4.1) according to a completely randomised design. The analysis tested for genotype, diet and genotype x diet interaction (Study 2) and farmlet and stocking rate (Study 3). All means are presented as least squares.

RESULTS

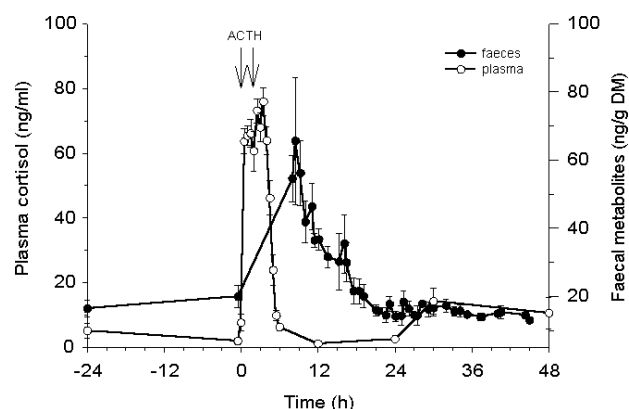
Serial dilution of bovine faecal extracts (1:2-1:128)

yielded a displacement curve parallel to the corticosterone standard curve. Net recovery of exogenous corticosterone (12.5-500ng standard) added to bovine faecal extract was assessed by calculating the linear regression between measured corticosterone and added mass ($r^2=0.99$; $y=5.831+1.102x$).

ACTH challenge: Plasma cortisol concentrations exhibited a 3.4-48 fold increase 30mins after ACTH injection and remained elevated for 4 to 4.5h (Figure 1). Plasma cortisol concentrations increased from 4.8 ± 1.4 ng/ml (pre-ACTH; mean \pm SEM) to 63.7 ± 3.9 ng/ml ($P<0.001$) 30mins after ACTH. Plasma cortisol concentrations peaked 2.5 to 3.5h after the initial ACTH administration (or 0.5 to 1.0h after the second ACTH injection) and had returned to basal levels at the end of the 6.5-h sampling session.

Basal faecal glucocorticoid metabolite concentrations were 17.3 ± 2.4 ng/g and had increased by the time the first samples were collected 7.5 to 8h after the initial ACTH injection (Figure 1). Faecal concentrations peaked between 8.0-9.5h after ACTH at 56.6 ± 8.0 ng/g ($P<0.05$; Figure 1). Metabolite concentrations remained elevated for 13h (range, 11.0-16.1h).

FIGURE 1: Mean (\pm SEM) plasma cortisol and faecal glucocorticoid metabolite concentrations in lactating dairy cattle after ACTH administration at time 0 and 2h (indicated by arrows).



HF genotype and diet: Mean faecal glucocorticoid concentrations of the cows fed Grass were consistently higher throughout the season (Table 1). There was also a significant genotype effect, the NZ HF cows having higher FGM concentrations than OS HF cows at all stages of the season except late lactation (Table 1). For both feeding regimes, concentrations of FGM were lowest during early lactation for both genotypes. For the grass-fed cows the metabolites were highest in mid lactation, whereas the concentrations increased over the whole lactation for TMR-fed cows. There was a significant genotype x diet interaction in late lactation (Table 1).

Stocking rate: Faecal glucocorticoid concentrations of the cows in the MID stocking rate (3.2 cows per hectare) were higher than the two other herds during the non-lactating (dry) and early lactation period (Table 2), but not different during mid and late lactation. Faecal glucocorticoid metabolites were highest during the dry period (June).

TABLE 1: Mean faecal glucocorticoid metabolites (ng/g) of Holstein Friesian (HF) from New Zealand (NZ) and overseas (OS) grazing pasture (Grass) or fed total mixed ration (TMR) during four stages of lactation.

Genotype Diet	NZ HF		OS HF		SED	Genotype	P Diet	G x D
	Grass	TMR	Grass	TMR				
Lactation								
Early	8.27	6.50	7.49	5.50	0.558	0.026	<0.001	0.943
Peak	16.6	9.74	13.2	8.02	0.982	<0.001	<0.001	0.228
Mid	19.2	12.9	13.5	8.62	1.658	<0.001	<0.001	0.549
Late	16.3	15.4	10.7	17.9	1.455	0.203	0.013	<0.001

TABLE 2: Mean faecal glucocorticoid metabolites (ng/g) of New Zealand dairy cattle grazing pasture at three stocking rates sampled during four stages of lactation.

Stocking Rate (cows/ ha)	LOW	MID	HIGH	SED	P
	2.2	3.2	4.3		
Lactation					
Dry	17.6	22.7	17.6	1.44	<0.001
Early	8.79	13.3	10.3	0.813	<0.001
Mid	11.3	9.94	11.6	0.733	0.064
Late	10.6	10.5	9.51	0.803	0.343

DISCUSSION

New Zealand dairy cows are farmed under pastoral systems that differ markedly from Europe and North America. A growing public awareness of animal welfare and interest in food production systems has created a demand for simple, less invasive measures of animal stress. Traditionally, plasma cortisol has been used to assess animal stress. This study has established that the measurement of immunoreactive glucocorticoid metabolites excreted in faecal samples can be used to monitor adrenal activity in dairy cattle. Tests for parallelism and quantitative recovery of corticosterone in cattle faecal extracts indicated that the cross-reactivity of the corticosterone antibody was sufficient to reliably measure glucocorticoid metabolites in bovine faeces. The ICN corticosterone antibody has been used to detect adrenal activity in a wide range of mammalian and avian species (Graham & Brown, 1996, 1997; Creel *et al.*, 1997; Wasser *et al.*, 1997, 2000; Monfort *et al.*, 1998; Goymann *et al.*, 1999, 2000).

Faecal glucocorticoid concentrations were significantly elevated in the first sample 8h after the initial administration of i.v. ACTH and reflected the elevation in plasma cortisol concentrations (4–5h). It is likely that the metabolites would have been detected in faecal samples before 8 hours but the digesta passage rate was faster than anticipated. The time lag between elevated plasma concentrations and the detection of metabolites is assumed to be roughly equivalent to the time for digesta to pass between the bile duct and the rectum (Palme *et al.*, 1996). Previous reports for ruminants suggested time lags of 8–16h in cattle (Palme *et al.*, 2000), 12h in sheep (Palme *et al.*, 1996), 14–20h in roe deer (Clauss *et al.*, 2000), and 22h in elk (Wasser *et al.*, 2000). The shorter time lag in this study probably is due to higher forage digestibility (spring pasture) and higher feed intake of lactating dairy cows. Events or conditions known to affect digesta passage (feed intake, forage composition and digestibility) and gut motility (withholding feed, medication, anaesthesia) need to be considered when deciding sampling protocols.

There are limited reports documenting the field application of faecal glucocorticoid measurement (African

Wild dogs, Creel *et al.*, 1997; Northern spotted owls, Wasser *et al.*, 1997; Spotted hyenas, Goymann *et al.*, 1999, 2000) and these have primarily monitored the glucocorticoid response to acute stressors such as translocation, aggression and human disturbance. Similarly we have measured elevated faecal glucocorticoid metabolites in cattle in response to transport and/or translocation to an unfamiliar environment (Morrow, unpub.). In the current study, we have described FGM concentrations in a large number of dairy cows maintained under a wide range of farm management systems. Glucocorticoid metabolite concentrations were influenced by genotype, feeding regime, stage of lactation and stocking rate. However, observed differences were relatively small compared to those following ACTH administration (Fig. 1) and transport (Morrow, unpub.). In addition, the difference in faecal dry matter (DM), as a measure of faecal composition, in cows fed TMR (16–21% DM) vs. grass (10–14% DM), cannot be excluded as a source of variation in FGM concentrations. In Study 2 faecal DM did not differ between genotypes or season. This suggests that differences in faecal DM were not associated with the small differences observed in FGM between genotype. Further research is required to determine the impact of these factors on animal stress and welfare.

There is little doubt that cortisol is a useful measure for an animal's response to a short-term acute stressor (e.g., transport, restraint). However, it is generally agreed that it is more difficult to interpret cortisol data alone (plasma or faecal concentrations) as a measure of long-term chronic stress (e.g., disease, social status, under nutrition) because the HPA axis may downregulate in response to chronic stress resulting in low cortisol concentrations (Ingram, 2000). This difficulty may be overcome by challenging the HPA axis at the pituitary (corticotrophin releasing hormone challenge), adrenal (ACTH challenge) or at the axis (psychological challenge) level and measuring the subsequent adrenal response rather than cortisol concentration *per se*. However, the extra requirement for the administration of drugs and/or additional handling may impact on the stress monitoring itself.

The FGM concentrations measured across all the dairying systems tested in this study were lower than FGM concentrations observed in HF following an acute stressor (translocation, transport) (Morrow, unpub.). The five farming systems monitored in this study represented a very wide range of feeding levels and environments that encompass almost all types of dairying systems in New Zealand. In conclusion, the difficulty inherent in interpreting cortisol concentration alone presents a paradox for researchers and regulators requiring a simple, practical and non-invasive approach for on-farm assessment of animal stress and welfare.

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